Involvement of Proteins in Cloud Instability of Shamouti Orange [*Citrus sinensis* (L.) Osbeck] Juice

Ilan Shomer,* Tatiana Yefremov, and Uzi Merin

Department of Food Science, Agricultural Research Organization, The Volcani Center, P.O. Box 6, Bet Dagan 50-250, Israel

The present study provides evidence for the involvement of protein in cloud instability of natural orange juice. No heat-coagulable proteins were found in the serum. Insoluble cloud matter (ICM) was heat-flocculated following enzymatic pectin degradation (EPD). The degree of flocculation depended on temperature (from ~50 to 75 °C) and was highest at pH 3.5. The fresh juice contained about 6.5 and 1.8 mg mL^{-1} of ICM and alcohol-insoluble solids of the serum (AISS), respectively. The ICM and the AISS contained, respectively, proteins (182 ± 14 and 119 ± 3 μg mg^{-1}), galacturonic acid (37 ± 6.6 and 175 ± 1 μg mg^{-1}), and neutral sugars (350 ± 44 and 338 ± 22 μg mg^{-1}). EPD resulted in removal of a marked portion of the pectin and was accompanied by partial removal of neutral sugars (mainly glucose and galactose) and some proteins from the pectic polymer in both AISS and ICM. Under electrophoresis, proteins of the AISS included bands in the range of 20−52 kDa and 10−14 kDa and those of the ICM at 22 and 50 kDa.

Keywords: *Citrus; cloud; neutral sugars; orange juice; pectin; pectinesterase; proteins*

INTRODUCTION

Orange juice cloud is composed of several colloidal fractions, such as oil microdroplets, membranes, intracellular organelles, pigment bodies (plastids), hesperidin crystals, and complexes of these colloidal bodies with proteins (Merin and Shomer, 1984; Shomer, 1988; Shomer et al., 1985).

Cloud instability is a severe problem in natural orange juice, particularly in fresh juice. It is related to pectin methyl esterase (PME), which is the catalyzing agent for the pectin demethoxylation that results in the formation of a pectate gel that is considered to be calcium pectate (Baker, 1980; Bruemmer, 1980; Joslyn and Pilnik, 1961; Sinclair, 1984; Thakur et al., 1997). It is common to heat treat the juice immediately after squeezing it from the fruit to inactivate the enzymes. This process reduces the formation of pectate gels, which leads to clarification.

Formation of the pectate gel is a process that takes place in the presence of Ca^{2+} ions or of sugar and acid (Thakur et al., 1997). This phenomenon is explained as a process in which Ca^{2+} ions interact with negatively charged sites of the pectic polymer exposed by demethoxylation. The formation of the calcium pectate has been shown to occur in both cell wall and plant tissue extracts (i) in a junction zone that interweaves with cellulose microfibrils in plant cell walls by means of either Ca^{2+} or noncellulosic polysaccharides (Fry, 1986; Morris et al., 1982; Powell et al., 1982; Rees and Wight, 1971); (ii) in the middle lamella that reinforces the intercellular adhesion (Shomer et al., 1990); and (iii) in a formation of pectate gel in plant tissue extracts and model solutions (Jarvis 1984; Morris et al., 1980, 1982). On the basis of the steric configuration of galacturonic acid units within the pectic polymers, it was suggested that the structure of calcium pectate gel appears as an "eggbox" model (Grant et al., 1973). However, apart from the importance of Ca^{2+} in the formation of pectate gel, it is not clear how the insoluble cloud constituents and particles become involved with the pectate gel in relation to the clarification process.

Proteins have been identified in specific parts of citrus fruits, such as juice sacs, pulp (Rockland, 1961; Sinclair, 1984), peel extracts, and their cloud particles (Shomer, 1988; Shomer et al., 1985) and in industrial processed reconstituted juice (Klavons and Bennett, 1985).

The role of proteins with regard to cloud instability has been studied in relation to three main aspects: (i) as enzymes (mainly PE) that were inactivated either by heat treatment or by proteases, which were considered to decrease enzymatic activity through the enzymatic decomposition of pectinases [the presence of protease was not found to restrain clarification (Biggs and Pollard, 1970; Castado et al., 1991; Krop and Pilnik, 1974)]; (ii) as complexes with other constituents such as phenols, tannic acid (Van Buren and Robinson, 1969; Van Buren and Way, 1978), hesperidin crystals, pectin, and unidentified components (Merin and Shomer, 1984; Shomer et al., 1985); (iii) in heat coagulation of soluble proteins, which are able to encapsulate and associate with cloud constituents, as has been shown for emulsified oily droplets, pigment constituents (Shomer, 1988), pectin, and neutral sugars (Shomer, 1991; Shomer et al., 1982). In addition, structural modifications of insoluble proteins were shown to occur as a result of heating (Shomer et al., 1987, 1998).

In peel extract (PEX), portions of the proteins have been found to be soluble and coagulable and to be involved with cloud instability. This was shown to occur through binding of the heat-coagulated soluble proteins to colloidal cloud constituents (Shomer, 1988; Shomer et al., 1985). The coagulation and the resulting cloud flocculation were affected by pH (Shomer, 1988). Each

* Corresponding author. Tel: +972 3 968 3706. Fax: +972 3 960 4428. E-mail: vtilan@netvision.net.il.

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of the protein fractions, fractionated according to coagulation/flocculation temperature and pH, is characterized by its own electrophoretic pattern and ultrastructure (Shomer, 1991; Shomer et al., 1991, 1995).

The effect of polysaccharides on the colloidal binding and coagulation of soluble proteins has been studied in model solutions (Imeson et al., 1977) and in tissue extracts (Shomer, 1988; Shomer et al., 1982). EPD of the PEX facilitates flocculation of heat-coagulable proteins, whereas without EPD the flocculation is withheld; from which it was concluded that high molecular weight pectin restrains the flocculation of heat-coagulable proteins (Shomer, 1988). The presence of nondegraded pectin, from both the albedo and the flavedo, caused finer ultrastructure in the coagulated proteins (Shomer et al., 1991). In a model system containing soluble potato proteins, it was shown that the addition of exogenous high molecular weight pectin suppressed flocculation of the heat-coagulable proteins and resulted in a delicate ultrastructure of the formed coagulum (Shomer et al., 1982). This phenomenon indicated a possible interaction between pectins and proteins, resulting in deposited heat-coagulated proteins on the pectic polymer.

The present study examined the capability of proteins to undergo coagulation in natural Shamouti orange juice, i.e., in "juice sac" extract and its role in cloud instability. Proteins, pectin, and neutral sugars in the insoluble cloud matter (ICM) and in the alcohol-insoluble solids of the serum (AISS) were analyzed with respect to EPD, pH, heat coagulation/flocculation, and cloud instability.

MATERIALS AND METHODS

Juice Preparation. Shamouti orange [*Citrus sinensis* (L.) Osbeck] juice was extracted from halved fruits with a hand-held electric squeezer. The oranges were cooled to 4 °C prior to squeezing in order to restrain PME activity in the extracted juice. The sectioned orange was gently pressed by hand onto the rotating squeezer head in order to prevent the possible extraction of the peel tissue. The juice was immediately filtered through four gauze layers into a flask immersed in a crushed ice slurry (0 °C). The flask with the cooled juice was immediately heat inactivated by immersing it in boiling water while stirring. By this process the juice was heated from 0 to 96 °C within 2−3 min and maintained at 90 °C for 5 min.

Enzymatic Treatment. Enzymatic pectin degradation was done by incubating the juice with pectinase [Ultrazyme-100 G Novo Ferment, Switzerland (consisting mainly of EC 4.2.2.10)]. This enzyme has been shown to degrade juice pectin (Merin and Shomer, 1999; Shomer, 1988). The enzyme was added to the juice in various concentrations and pH levels according to treatment and was incubated with gentle shaking at 36−40 °C for 60 min.

Flocculation was obtained by EPD of the juice and heating at 90 °C for 5 min (EPD heated). Five control treatments were carried out to assess the clarification due to heat coagulation: (1) Juice (control); (2) juice heated at 90 °C for 5 min (control heated); (3) juice after EPD (EPD); (4) juice after incubation with inactivated Ultrazym [a solution of 1% (w/v) Ultrazym in distilled water was boiled under reflux for 5 min, cooled to ambient temperature, and added to the juice in ratios adjusted to the required concentration (inactivated EPD)]; (5) juice after incubation with inactivated Ultrazym (same as 4) and heated at 90 °C for 5 min (inactivated EPD heated).

Heat Coagulation Treatments. A total of 10 mL of the treated juice was heated in a test tube of 1 cm diameter. The tubes were immersed in a water bath at the set temperature for 10 min or placed in an autoclave at 120 °C. The sample was then cooled rapidly in an ice bath to room temperature, transferred to ambient temperature, and held undisturbed for 60 min to allow flocculation. The uppermost 2 mL of the juice column from the tube were taken for flocculation intensity measurements. Turbidity was determined by the light transmittance measurement at 660 nm, using Spectronics 601 spectrophotometer (Bausch & Lomb, USA). To collect the scattered transmitted light the cuvette was placed with the opaque side facing the photocell, using the same quartz cuvette for all the measurements (Merin and Shomer, 1984; Shibata et al., 1954).

pH Adjustment. Cloud flocculation of the juice was determined at various pH levels between 1.5 and 6.5. Lowering or increasing the pH was done by addition of either concentrated HCl or 30% aqueous NaOH solution, respectively, into the juice with vigorous stirring. All the juice samples were brought to the same total volume prior to the coagulation treatment.

Determination of ICM, AISS, Pectin, Neutral Sugars, and Proteins. To obtain ICM for chemical analyses, the juice was ultracentrifuged at 27000*g* for 15 min. The precipitate, i.e., the ICM, was redispersed in distilled water, stirred, and centrifuged three times, freeze-dried, and weighed to determine the dry matter content in the juice. The AISS was separated from the supernatant, i.e., the serum, by adding analytical EtOH up to 80% (v/v). The formed AISS was separated by centrifugation at 15000g for 15 min. The precipitate was redispersed in aqueous solution of 80% EtOH, stirred, and centrifuged three times to remove the residues of the low molecular weight solutes. The precipitate was freeze-dried and weighed to determine its dry matter content in the juice.

Proteins, pectin (as galacturonic acid), and neutral sugars were determined in both the ICM and in the AISS in triplicates. Pectin, as galacturonic acid, was analyzed according to Ahmed and Labavitch (1977). Protein content was determined by the Kjeldahl method. Proteins of both the AISS and the ICM were characterized by loading a sample that included ∼75 of mg protein (calculated from the Kjeldahl analysis) on SDS−PAGE as described by Shomer et al. (1991, 1995).

Neutral sugars were analyzed according to Blakeney et al. (1983). About 10 mg of dry matter were hydrolyzed in 125 μL of 72% H_2SO_4 at room temperature for 45 min. A total of 1.35 mL of distilled water was added, and the sample was autoclaved at 121 °C for 1 h and then neutralized with 320 μL of 15 M ammonia. As internal standard, 25 μL of myo inositol was added to 0.1 mL of the neutralized sample. The monosaccharides were reduced by adding 1 mL of the sodium borohydride solution (2 g in 100 mL of anhydrous dimethyl sulfoxide) and heating at 40 °C for 90 min. Excess sodium borohydride was decomposed by addition of 0.1 mL of 18 M acetic acid. Alditol acetates were obtained by acetylation by mixing the reduced monosaccharides with 0.2 mL of 1-methylimidazole and 2 mL of acetic anhydride. Five milliliters of distilled water was added after 10 min at room temperature, and 1 mL of dichloromethane after an additional 20 min. The mixture was mixed vigorously and left stationary to allow separation of the alditol acetates in the dichloromethane phase. The alditol acetates dissolved in dichloromethane were analyzed according to Blakeney et al. (1983) by means of gas liquid chromatography (Hewlett-Packard 5790A series gas chromatograph) with a flame ionization detector. The solution to be analyzed was injected into a column of 3% SP-2340 on a Supelco Port 100/120 mesh under the following conditions: the column temperature was increased from 190 to 270 °C at a rate of 4 °C min^{-1}; injection and detector temperatures were 250 and 300 °C, respectively; nitrogen was used as the carrier gas at a rate of 24 mL min^{-1}; air and hydrogen were used for the combustion at rates of 300 and 30 mL min^{-1} respectively.

Statistical Analysis. The experimental data were analyzed according to the Friedman two-way ANOVA by ranks (Siegel, 1956) as nonparametric statistics. This method was used since in some cases triplicates were not performed. Protein, pectin,

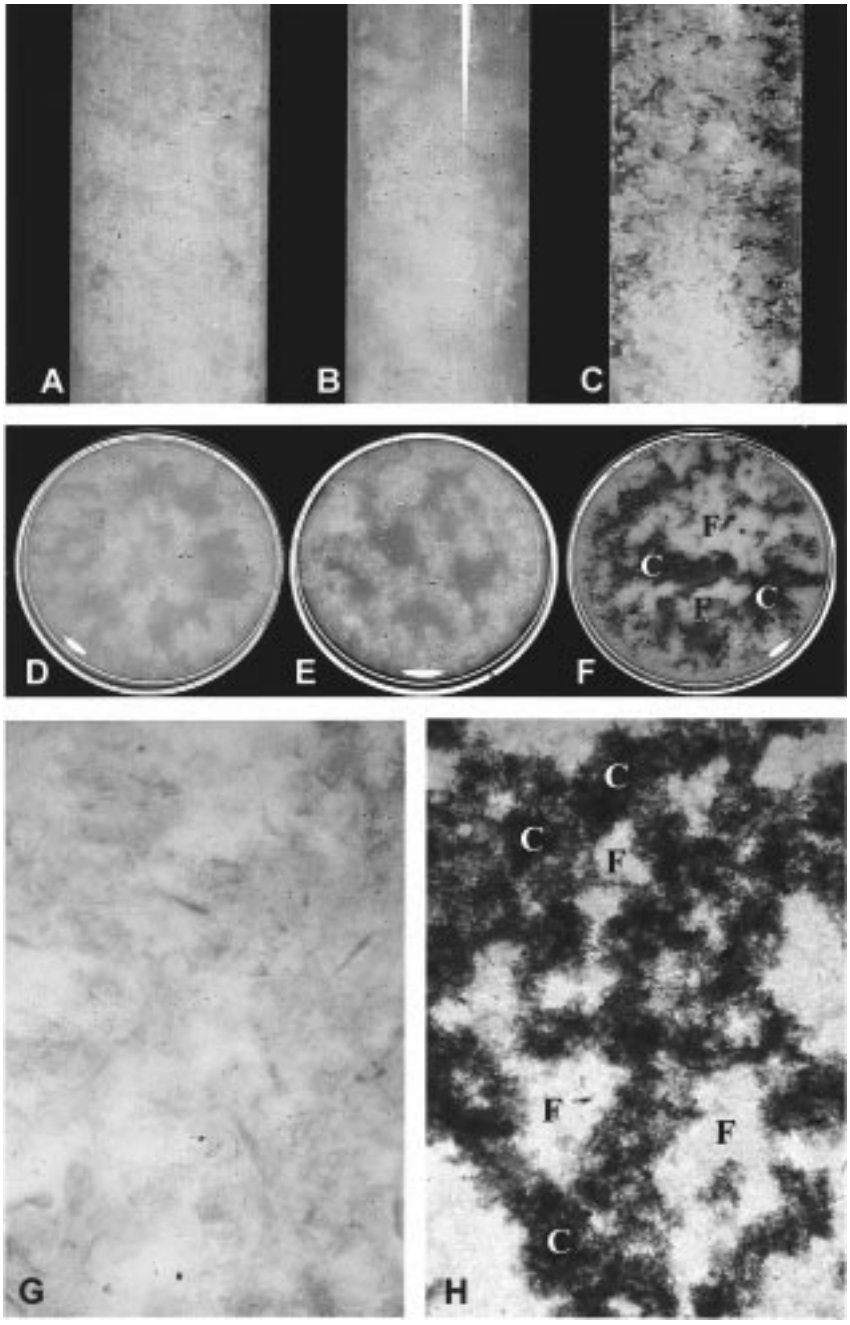

Figure 1. Appearance of natural Shamouti orange juice during heating. (A) Heated juice in a column at 90 °C for 2 min and cooled to reach ambient temperature. (B) Enzymatic degraded juice in a column at ambient temperature. (1C) Enzymatic pectin-degraded juice in a column, heated at 90 °C for 2 min and cooled to reach ambient temperature. (D−F) Same as A−1C, respectively, but in a Petri dish. (G and H) Stereomicroscopy micrographs of D and F, respectively (×100) [the micrograph of E is not shown because it was identical to that of D]. Letters in F and H: C, region of clarified serum; F, region of flocculated cloud matter.

and neutral sugars were analyzed in triplicates. Error bars indicate standard deviation (the absence of error bars indicate ±0 SD).

RESULTS AND DISCUSSION

Figure 1 presents a typical appearance of clumps of heat-flocculated cloud. When heating was exerted on the control juice column (Figure 1A), the cloud seemed quite stable. A similar view but with moderate flocculation is seen in a juice column after EPD without heating (Figure 1B). In comparison to the control treatments, visible clumps and regions of clarified serum were observed to develop in EPD juice a short time after the beginning of the heating process with initial stages of cloud settling (Figure 1C). More detailed appearance of cloud flocculation is seen in a thin layer (2−4 mm thickness) of juice column (Figure 1D−F). These observations enable characterization of the flocculation while eliminating the mutual effects of loaded gravitational force that the settled cloud clumps exert on each other. Stereomicroscopy observations show the detailed structure of the cloud in heated juice (Figure 1G) and of the clumps in the heated EPD juice (Figure 1H). Obvious regions of clarified serum are observable between the flocculated cloud clumps (Figure 1F,H).

The effect of EPD and heating on cloud flocculation is shown in Figure 2. It appears that this treatment led to accelerated precipitation and to reduced turbidity of

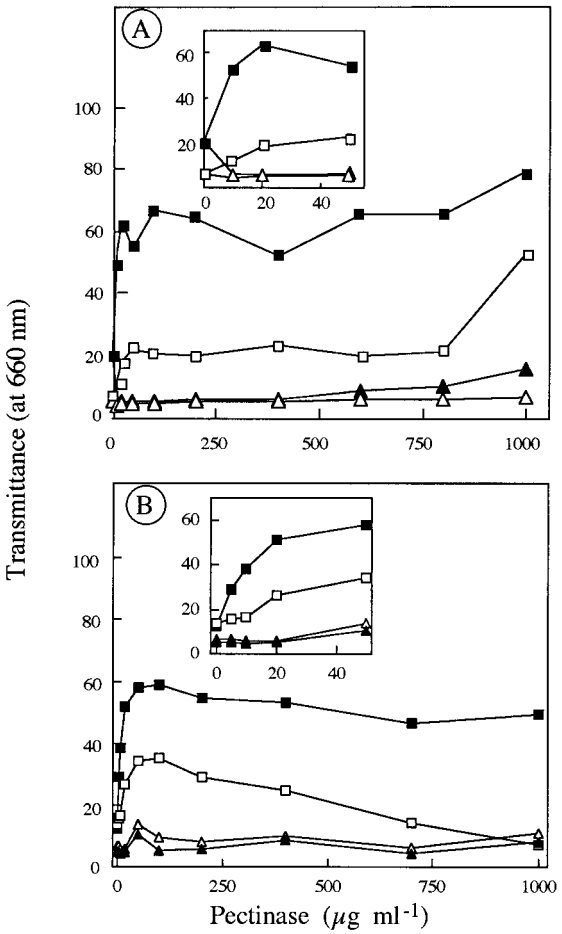

Figure 2. Turbidity of the upper zone of natural Shamouti orange juice as affected by heat coagulation/flocculation as a result of enzymatic pectin degradation. Treatments marked by different letters are significantly different ($p < 0.05$) according to Friedman ANOVA. (A) Juice from fruits harvested in mid-December; (B) juice from fruits harvested in April: △, inactivated EPD[a]; ▲, inactivated EPD heated[a]; □, EPD[b]; ■, EPD heated[c]; Insert: Low concentrations of pectinase.

the upper zone of the juice column, even at low pectinase concentrations of 10−30 μg mL^{-1} (Figure 2). Heating of the same juice without EPD or following heat-inactivated EPD did not result in rapid clump formation and cloud flocculation at all the treated enzyme concentrations. The nonheated EPD juice in the average was somewhat less stable than the other control treatments (Figure 2). Differences in the extent of clarification were found between juice of oranges harvested in mid-December (Figure 2A) and those harvested in April (Figure 2B).

Examination of the cloud clump formation in the heated EPD juice and of its precipitation revealed a distinct clarification at pH 3.5 in the juice of oranges harvested in mid-December (Figure 3A). It is worth noting that, in the juice from oranges harvested in April, the nonheated EPD juice was clarified similarly to that of the heated one (Figure 3B). In the present study, when the juice was heated to various temperatures between ambient and 125 °C, a marked clarification was noted at temperatures over 50 °C but only in the EPD-treated juice; clarification increased to a maximum at temperatures of 75 °C and lowered at 100 °C (Figure 4). Clarification under various heating temperatures of EPD juice from fruit harvested in April (Figure 4B) was lower than that of December (Figure 4A). This trend of

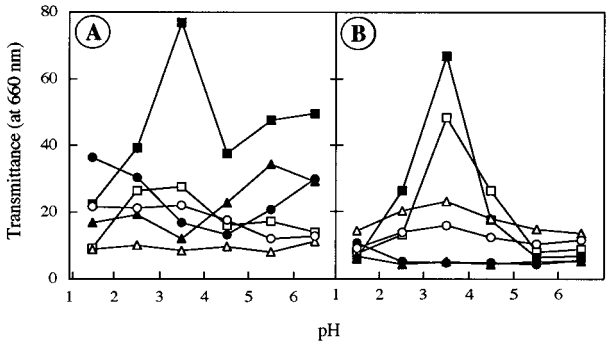

Figure 3. Turbidity of the upper zone of natural Shamouti orange juice as affected by heat coagulation/flocculation at various pH levels. Treatments marked by different letters are significantly different ($p < 0.05$) according to Friedman ANOVA. (A) Juice from fruits harvested in mid-December: ○, control[a]; ●, control heated[a]; △, inactivated EPD[a]; ▲, inactivated EPD heated[a]; □, EPD[a]; ■, EPD heated[b]. (B) Juice from fruits harvested in April: ○, control[a]; ●, control heated[a]; △, inactivated EPD[b]; ▲, inactivated EPD heated[b]; □, EPD[c]; ■, EPD heated[d].

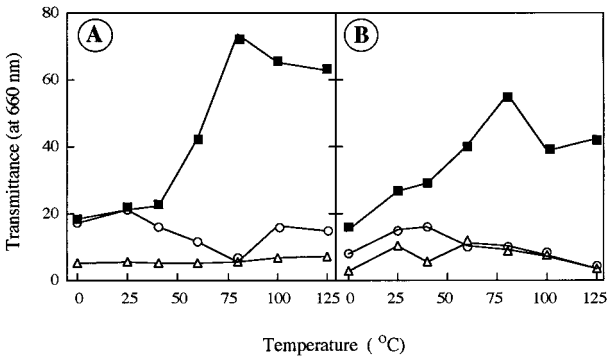

Figure 4. Turbidity of the upper zone of natural Shamouti orange juice as affected by heat coagulation/flocculation at various heating temperatures. Treatments marked by different letters are significantly different ($p < 0.05$) according to Friedman ANOVA. (A) Juice from fruits harvested in mid-December: ○, control[a]; △, inactivated EPD[b]; ■, EPD heated[c]. (B) Juice from fruits harvested in April: ○, control[a]; △, inactivated EPD[a]; ■, EPD heated[b].

higher clarification in juice from fruits harvested in April is consistent with the results of the effect of enzyme concentration (Figure 2) and pH (Figure 3). It can be hypothesized that the nonheated EPD juice of April is from overripe fruits where some of the proteins are partially denatured. These cloud proteins are more sensitive to flocculation, similar to those of juice from overripe Valencia orange fruits (Shomer et al., 1999).

Cloud instability is not identical in juices extracted from fruits of different harvesting batches. An example for such variability was shown by Shomer et al. (1999). Marked differences in juice stability are sometimes seen between orchards, harvest times, and storage duration, both in heat-inactivated and in fresh juice. Sometimes untreated fresh juice remains relatively stable, and sometimes heat-treated juice is unstable to a degree, which renders its appearance unacceptable.

These results are consistent with previous findings on cloud formation through protein coagulation by heat, which are soluble in the case of flavedo PEX, and whose flocculation is facilitated by EPD (Shomer, 1988, 1991; Shomer et al., 1991) and moderated by added soluble pectin (Shomer et al., 1982). Unlike the case of PEX serum, in the juice the proteins are part of the cloud; therefore, no flocculation of heat-coagulable soluble

proteins occurred in the serum of natural juice, either before or after EPD.

It should be explained that the intensity of heat coagulation of soluble proteins could be evaluated in both clear aqueous solutions and turbid liquids. In clear solution, the coagulation is clearly recognized by the development of turbidity, which can be measured immediately in a mixed suspension (before settling). The turbidity is positively correlated with protein concentration and their sensitivity to heat coagulation (Shomer 1988; Shomer et al., 1982, 1991, 1995). Coagulation of protein in aqueous opaque liquids cannot be visually identified through turbidity evolution. In such cases, the occurrence of protein flocculation is manifested by two means: (1) clarification due to formation of clumps and their rapid precipitation (Shomer et al., 1991) and (2) formation of deposits of coagulated protein on the surfaces of the cloud particles (Shomer, 1988).

Since the juice does not contain soluble proteins that are heat coagulable, it is impossible to evaluate its flocculation by turbidity evolution in the juice serum. In this case, the involvement of protein flocculation in clarification may be detected in pH and temperature conditions under which the juice clarifies and proteins are typically coagulated and flocculated. Moreover, at temperature levels where pectin is more soluble and PME is inactivated, cloud flocculation and clarification are enhanced. These circumstances suggest that cloud instability in natural juice involves protein interactions with various components such as cloud particles and essential oils (Shomer, 1988), cell wall fragments (Thakur et al., 1997), and pectin (Takada and Nelson, 1983).

Heating following natural PME action results in much enhanced interaction of demethoxylated pectin with proteins (Shomer, 1991) and, in the case of natural juice, with the cloud proteins. Such an association enhances cloud flocculation in cases in which the juice underwent EPD prior to concentration or filtration. This could also be the case when heat is applied (in addition to the first heating immediately following juice extraction) for inactivation of added pectinase and pasteurization of reconstituted juices.

In the presence of both high molecular weight soluble pectin and soluble proteins, upon heating, coagulated proteins appear as matter deposited onto the pectic polymer. This phenomenon has been observed both in model solutions (Imeson et al., 1977; Shomer et al., 1982) and tissue extracts (Shomer, 1988; Shomer et al., 1995). Unlike the above, in the case of natural juice, where the proteins are part of the insoluble cloud constituents, it is likely that upon heating cloud components are deposited onto other pectic polymer of the cloud. Under such conditions, cloud flocculation is mild since the high molecular weight (relatively methoxylated) pectins moderate the mutual interactions of the cloud constituents.

Cloud and AISS Proteins. The ICM and the AISS account for about 6.5 and 1.8 mg mL^{-1}, respectively, of the fresh juice. Analyses of the ICM revealed the presence of ~1187 ± 91.2 and 1139 ± 50 μg mL^{-1} protein before and after EPD, respectively. The ICM dry matter contained 182 ± 14 and 205 μg mg^{-1} before and after EPD, respectively.

Analyses of the AISS revealed the presence of ~215 and 55.4 ± 6.3 μg mL^{-1} protein, before and after EPD, respectively. AISS dry matter included 119 ± 3 μg mg^{-1} protein, either before or after EPD (Figure 5). The cloud protein content is in agreement with reported data for reconstituted orange juice (Klavons et al., 1991) and somewhat higher than those reported for commercial orange and lemon juice (Klavons and Bennett, 1985; Klavons et al., 1991). EPD did not result in a significant change of protein content in the ICM, but in the AISS, less than 50% of protein than that of the non-EPD juice were found. It is possible that a certain amount of the nitrogenous matter comprises pectin-bound amino acids or peptides that associate with the coagulated proteinaceous matter upon heating. When the juice is subjected to EPD, the pectin-bound proteinaceous matter would be released and partially washed away during preparation. Traces of Ultrazym proteins are seen in the AISS of the EPD juice of the Shamouti orange (Figure 6) but not clearly in that of the Valencia orange (Shomer et al., 1999). It can be assumed that the soluble pectin and neutral sugars of the AISS in juice of Shamouti orange (Figure 6) bind higher amounts of Ultrazym protein than that of the Valencia (Shomer et al., 1999).

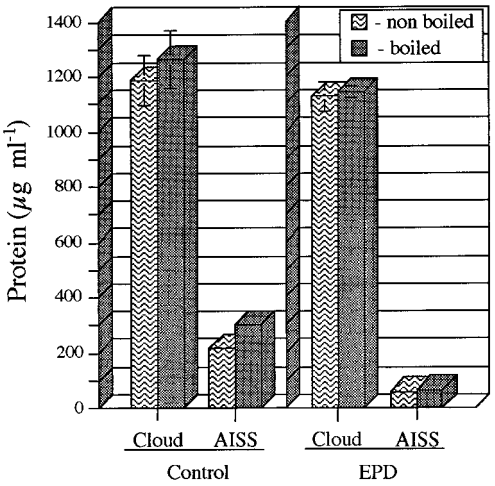

Figure 5. Protein content of insoluble cloud matter (ICM) and alcohol-insoluble serum solids (AISS) of natural Shamouti orange juice untreated and after enzymatic pectin degradation (EPD) and heat coagulation/flocculation. Error bars indicate ± SD of triplicates.

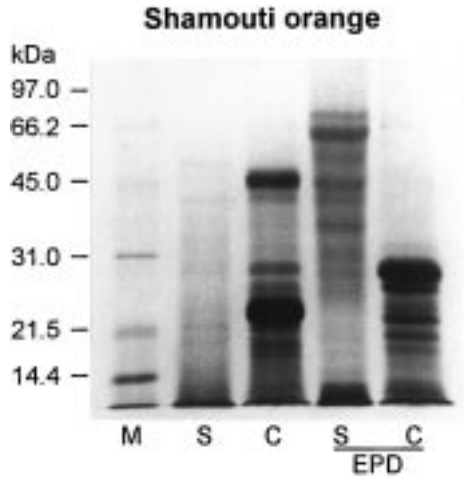

Figure 6. Electrophoresis profiles of proteins of insoluble cloud matter and alcohol-insoluble serum solids of natural Shamouti orange juice after heating at boiling temperature and enzymatic pectin degradation. Molecular weight markers; EPD, enzymatic pectin degraded juice; C, insoluble cloud matter of pectinase degraded juice; S, alcohol-insoluble serum solids.

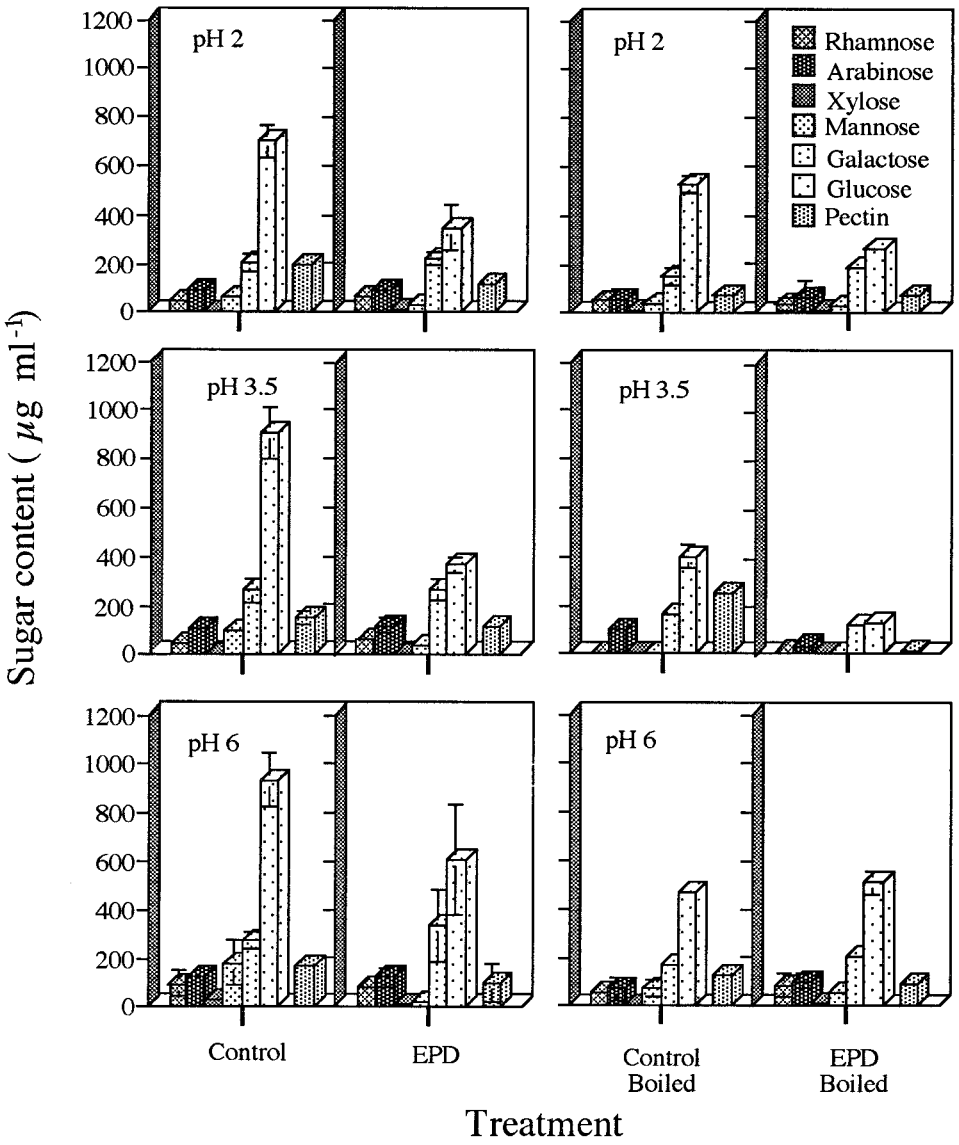

Figure 7. Contents of galacturonic acid and neutral sugars in insoluble cloud matter of natural Shamouti orange juice at three pH levels [2, 3.5 (natural level), and 6] and after heat coagulation/flocculation and enzymatic pectin degradation (EPD). Error bars indicate ± SD of triplicates.

Electrophoresis of the ICM proteins revealed polypeptides in the range of 17−50 kDa with distinct bands at 22 and 50 kDa. This profile include the same bands as that of Valencia orange (Shomer et al., 1999) but at different intensities. No residual exogenous pectinase was found in the ICM, which had undergone flocculation. Therefore, it is suggested that the cloud was not affected by the possible heat coagulation of the exogenous pectinase. The AISS proteins included polypeptides in the range of 20−52 kDa with relatively weak bands. SDS−PAGE analysis of Ultrazym itself showed polypeptides in the range of 31−67 kDa. Hence, it was identified that the AISS of the EPD juice included four additional bands of the Ultrazym proteins in the range of 45−65 kDa.

Although polypeptides of molecular weight over 30 kDa were not found in the electrophoretic pattern of the ICM, following EPD (Figure 6), the average content of total cloud proteins was reduced by 5 and 9.5%, respectively, which is not significant (Figure 5). It is possible that some of the cloud proteins are bound to a relatively high molecular weight pectin. EPD results in degradation of the pectic polymer and its associated neutral sugars, leaving the polypeptides with lower sugar content. Thus, the disappearance of the higher molecular weight bands in the SDS−PAGE profile may be due to abnormal SDS binding, shape, and charge of the glycoproteins before the enzymatic treatment (See and Jackowski, 1990). Degradation of the insoluble cloud protein by residual proteases from the Ultrazyme is uncertain but has to be considered since the protein content in the ICM did not change. Moreover, cloud flocculation of heat-treated EPD juice had already appeared at very low Ultrazyme concentrations (Figure 2), at which the protease effect would not be detectable even during 60 min of incubation. However, the possible involvement of residual protease from the added pectinase, which is insufficient to completely degrade proteins to soluble peptides but enough to lower their molecular size, deserves further elucidation. It should be mentioned that other studies (Shomer, 1988; Shomer et al., 1991, 1999) and the control treatments in the present study showed that the Ultrazyme proteins did not affect stability on their own coagulation since heat-inactivated Ultrazyme did not cause flocculation.

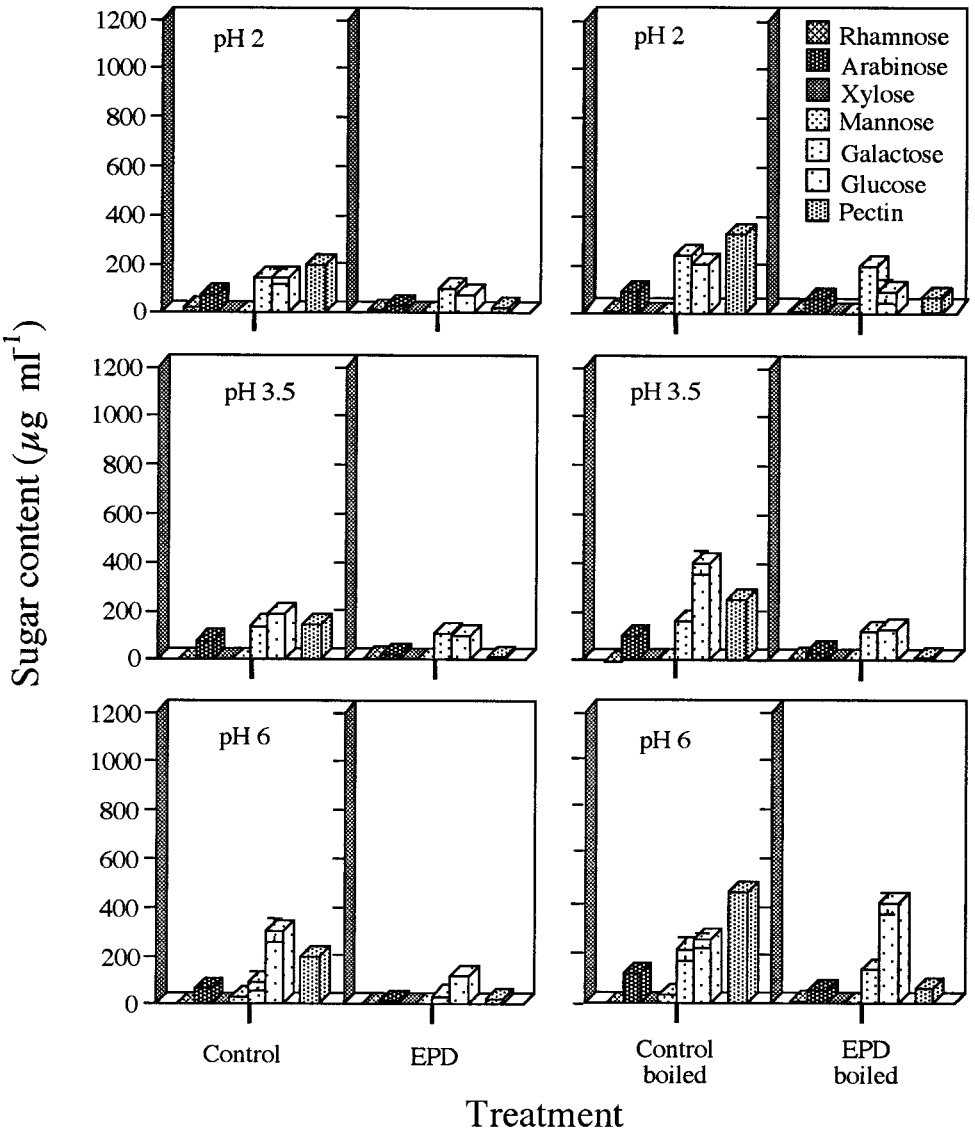

Figure 8. Contents of galacturonic acid and neutral sugars in the alcohol-insoluble serum solids of natural Shamouti orange juice at three pH levels [2, 3.5 (natural level), and 6] and after heat coagulation/flocculation and enzymatic pectin degradation (EPD). Error bars indicate ± SD of triplicates.

Similar SDS−PAGE profiles were found between the electrophoretic profiles of heated and nonheated juice of both the ICM and the AISS (Shomer et al., 1999), either before or after EPD.

Pectin and Neutral Sugars. Polysaccharides of both the serum and the ICM include pectin (analyzed as galacturonic acid) and the neutral sugars rhamnose, arabinose, xylose, mannose, galactose, and glucose (Figures 7 and 8). The galacturonic acid content in the AISS and in the ICM is 175 ± 1 and 37 ± 6.6 μg mg^{-1}, respectively. The total neutral sugars content in the AISS and in the ICM is 338 ± 22 and 350 ± 44 μg mg^{-1}, respectively. The AISS content in the juice (1.8 mg mL^{-1}) is much lower than that of the ICM (6.5 mg mL^{-1}); thus, the total amount of galacturonic acid in the AISS (~143 μg mL^{-1}) is similar to that of the ICM (~152 μg mL^{-1}). Glucose and galactose are the predominant neutral sugar in the AISS (187 and 139 μg mL^{-1}, respectively) and are found in much higher relative quantities in the ICM (907 and 261 μg mL^{-1}, respectively). These relative amounts are different from those found previously in the PEX (Shomer, 1991), in which the predominant sugars in the AISS were glucose and arabinose (258 and ~40 μg mL^{-1}, respectively, and in the coagulates, 21.6 and 10.5 μg mL^{-1}, respectively).

EPD was accompanied by reduction of ICM glucose (to ~366 μg mL^{-1}) and of glucose and galactose (to 100 and 138 μg mL^{-1}, respectively) in the AISS. These results are in agreement with those of the PEX, in which the amount of part of the neutral sugars (mainly glucose) was found to be in accordance with the content of galacturonic acid.

Following heat treatment, the total amount of polysaccharides (pectin and neutral sugars) decreased significantly in the ICM from 1055 to ~909 μg mL^{-1} in the control and from ~968 to ~298 μg mL^{-1} in the EPD juice (Figure 7). In contrary, heat treatment caused a significant increase in the amount of the AISS polysaccharides from 549 ± 33 to 909 ± 81 μg mL^{-1} in the control and from ~246 ± 10 to ~297 ± 58 μg mL^{-1} (not significant) in the EPD juice (Figure 8). It seems that as a result of heating, a certain amount of the polysaccharides (pectin and its neutral sugars) was partially removed from the ICM (perhaps by β-elimination) and appeared as AISS matter.

Previous ultrastructural studies showed that, in the presence of nondegraded soluble pectin, the heat-coagulable proteins seemed to be bound to pectic polymers. This binding determines the flocculation intensity and the ultrastructural patterns of the coagulated proteins (Shomer et al., 1982, 1991). Pectin and neutral sugars were found to be bound to heat-coagulated proteins, particularly in noninactivated PEX after EPD in which the active endogenous PME yielded higher portion of demethoxylated galacturonic acid units (Shomer, 1991). Previous studies (Klavons et al., 1991; Shomer, 1988, 1991; Shomer et al., 1982, 1991; Takada and Nelson, 1983) and the present findings support the assumption that the formation of pectate gels following PME activity may also result in the formation of a protein−pectate precipitate. This is either in addition to or in conjunction with calcium pectate. With respect to the eggbox theory (Grant et al., 1973; Takada and Nelson, 1983), involvement of proteinacous matter in the formation of a pectate gel deserves further study. From the point of view of natural juice, which includes cloud proteins, it can be assumed that PME activity would result in a higher association between demethoxylated pectin and cloud components. Hence, it can be assumed that the main reason for clarification as a result of PME activity is the binding of cloud components through their proteins to the pectate gel. Such binding is enhanced by heat coagulation of the cloud proteins following PME activity.

CONCLUSIONS

The experiments (heat-coagulation/flocculation, AISS and ICM protein, pectin and neutral sugars analyses) of the present study showed the formation of ICM clumps in conditions under which proteins tend to coagulate and flocculate (above 70 °C and at pH 3−4). Cloud flocculation and the resulting clarification was more intense in the pH range of 3−4 and was enhanced by EPD and heating. Under these conditions the pectin is more soluble, and the PME is less active (its optimal activity is at pH 7 and above) or inactive (at 90 °C). These facts and the fact that the cloud protein content ($\sim$182 μg mg^{-1}) was much higher than that of pectin ($\sim$37 μg mg^{-1}) suggest that clarification was a result of cloud protein coagulation/flocculation. It is suggested that PME activity increased the association between the pectin and the cloud proteins, leading to cloud protein−pectin flocculation. In this context, a previous study (Shomer et al., 1991) showed a much higher content of galacturonic acids in a protein aggregate when the fresh extract was incubated (before the heat coagulation) to facilitate PME activity. In light of the accepted approach that blames cloud instability to the formation of calcium pectate and the results of the present study, the possible connection between protein coagulation and PME deserves further elucidation.

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